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EPIGENETICS

Heterogeneous distribution of histone methylation in mature human sperm

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Abstract
Purpose To analyze the presence of various histone modifications in ejaculated human spermatozoa
Methods In this prospective study, seminal ejaculates from 39 normozoospermic individuals were evaluated for semen analysis and the presence of histone modifications in isolated nuclei.
Results We observed heterogeneous presence of histone methylation in normal mature human sperm. We observed that 12 to 30 % of the nuclei of normal sperm contain a heterogeneous distribution of the marks H3K4Me1, H3K9Me2, H3K4Me3, H3K79Me2, and H3K36Me3. Moreover, the presence of these marks is higher in the poor motile fraction of the ejaculate, which is associated with poor morphology and functional quality. In contrast, we did not observe histone acetylation (H3K4Ac and H4K5Ac) in normal or abnormal mature human sperm
Conclusions Defects in the process of spermatogenesis may alter the correct epigenetic programing in mature sperm. Further studies are required to evaluate the impact of these findings in human infertility

Keywords Epigenetic · Human sperm · Histone modifications · Sperm subpopulations

Capsule Histone modifications in human sperm.

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Introduction

The establishment and maintenance of the germ cells requires a fine regulation of epigenetic marks starting in the primordial germ cell population [1]. During spermiogenesis, most of the canonical histones are exchanged for protamines that tightly pack DNA in mature sperm [2]. It was reported that about 4–15 % of the human sperm genome is packed as nucleosomes [3], consisting of canonical or variant histone proteins. Historically, their presence in mature sperm has been associated to an insufficient replacement of histones by protamines with no impact in future embryo development. However, recent evidence using high-resolution genomic approaches has clearly demonstrated a programmatic retention in particular genes/loci that are important for embryo development [4]. This discovery opens the possibility of other potential sources of male infertility largely unexplored. Hammoud and coworkers [5] recently reported that some alterations in histone retention and epigenetic modifications at developmental and imprinted gene loci existed in the sperm of infertile men when compared to fertile donors.

Most of the causes of male infertility still remain undiagnosed or classified as idiopathic because of an incomplete evaluation of sperm function due to the lack of the appropriate tests. For example, the evaluation of the sperm nucleus and DNA is limited to the analysis of its protein composition (amount of protamines or histones) or the integrity of the DNA [6]. There are almost no reports in the literature that address the potential effects of sperm histone content and post-translational modifications on fertility and embryo development. Histone modifications are one of the most thoroughly studied areas in epigenetics, mainly because of their importance in transcriptional regulation of gene expression. Because little is known about the presence and distribution of different histone post-translational modifications in ejaculated human sperm, we aimed to characterize this event in normal and abnormal spermatozoa.

Materials and methods

Semen specimens and sample processing

Normozoospermic semen specimens according to World Health Organization [7] were obtained from 39 normal healthy volunteers after informed consent. The study protocol was approved by the IBYME Institutional Review Board. Specimens were used solely for research and not for clinical purposes. Donor data were kept confidential. Samples were collected by masturbation after 36–48 h of abstinence, allowed to liquefy at room temperature and processed within 1 h of collection. For nuclei isolation, samples were washed twice by centrifugation at 300 g for 10 min in human tubal fluid (HTF) and resuspended in 1 ml PBS. In other experiments, semen samples were loaded onto a 45 and 90 % discontinuous Isolate gradient (Irvine, USA) gradient, as previously described [8]. The resulting interfaces between the layers of 45 and 90 % (L45) and the 90 % pellet (L90) were aspirated and transferred to separate tubes. Sperm suspensions were then diluted with PBS and processed for nuclei isolation. In other sets of experiments, mouse germ cells, were isolated as previously described [9]

Sperm nuclei isolation

Human sperm nuclei were isolated according to the protocol modified from Yebra and Oliva [10]. Semen was washed three times by centrifugation at 1600 g for 10 min in 50 mmol/l Tris–HCl, pH 7.2, and 0.15 mol/l NaCl (10× sample volume). The sperm pellet was resuspended in 2.6 ml of the same buffer containing 1 % SDS, incubated for 15 min at room temperature and the spermatozoa sonicated (6×15 s at 200 W) with a Branson sonifier cell disruptor, model W140 (Branson Sonic Power Co., Plainview, NY, USA). The sonicated cells were separated in two equal aliquots, each of which was placed on top of 4 ml 1.1 mol/l sucrose in 50 mmol/l Tris–HCl, pH 7.2, and centrifuged at 3500 g for 1 h. Pellets were recovered and washed twice by centrifugation at 1600 g for 10 min in 50 mmol/l Tris–HCl, pH 7.2. Lack of contamination of the nuclear fraction obtained with sperm tails was tested by microscopical observation.

Immunocytochemistry of isolated sperm nuclei

Immunocytochemistry was performed according to the protocol described by Zahn and coworkers [11]. Isolated sperm nuclei were washed in PBS and air-dyed onto a glass slide. Cells were fixed with 4 % paraformaldehyde in PBS during 20 min and permeabilize with 0.1 % Triton X-100 during 20 min at room temperature. Cells were then blocked with 1 % goat serum during 45 min at room temperature. The slides were incubated overnight at 4 °C with primary antibodies against H3K4Me, H3K4Me3, H3K9Me2, H3K36Me3 (Cell Signaling) H3K79Me2 and H3K4Ac (Active Motif) or H4K5Ac (Abcam), diluted 1:100 (50 mg/ml) in blocking buffer in a humidified chamber. After washing tree times with PBS, slides were incubated with goat anti-rabbit IgG conjugated with Alexa488 (Molecular Probes), diluted 1:300 in blocking buffer during 60 min at room temperature in a humidified chamber. Following incubation, slides were washed three times with PBS, air-dried and mounted with Vectashield (Vector, USA). Sperm were examined using a confocal microscope Nikon C1 (USA). At least 200 cells were counted in different fields and the percentage of nuclei showing fluorescence was calculated. Negative controls were performed by omitting the primary antibody.

Statistical analysis

Statistical analysis was carried out by a paired *Student's t test after* the data were normalized by an *arcsin* transformation using the GraphPad Prism program (GraphPad software). All tests were two-tailed with statistical significance assessed at the $P<0.05$ level.

Results

To analyze histone post-translational modifications persisting in nucleosomes of mature human sperm, we performed immunocytochemistry using isolated nuclei. All antibodies used to immunolocalize the modifications (H3K4Me, H3K4Me3, H3K9Me2, H3K79Me2, H3K36Me3, H3K4Ac and H4K5Ac) were previously validated for specificity of chromatin-associated staining using Hela cells (data not shown). After nuclei isolation and treatment with Triton X-100, it was not necessary to decondense sperm nuclei using DTT or other agents to allow the antibodies to gain access to the specific epitopes since by using immunofluorescence analysis we obtained similar results with or without decondensation (not shown). As a control, we also validated our procedure using anti-histone H3 and anti protamine 1 and 2 which are well known components of the mature sperm nuclei (data not shown).

First, we evaluated the presence of histone acetylation (H3K4Ac and H4K5Ac) in ejaculated human sperm. In this set of experiments, we did not observe histone acetylation in ejaculated human sperm (Fig.1a). As a control, we performed immunofluorescence using the aforementioned anti-acetylated histone antibodies in isolated mouse spermatids at different stages of differentiation to validate our protocol because of the difficulties in obtaining these cells from human testis. As observed by other authors, we observed histone acetylation H4K5Ac in round spermatids but not in elongated spermatids (Fig.1b).

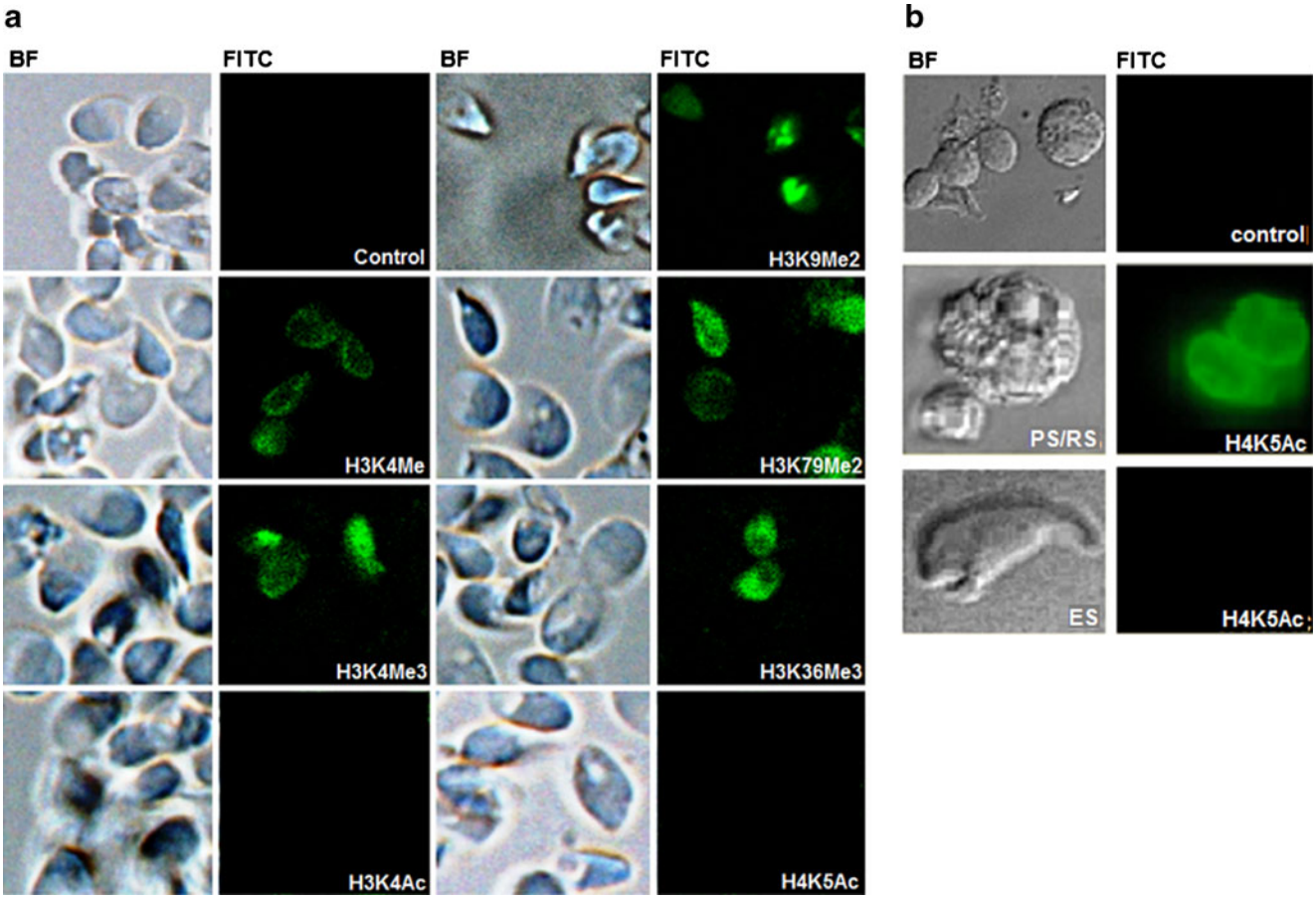

Fig. 1 **a** Immunofluorescence staining of human sperm nuclei for the different histone marks assessed in this study. *Right panel*, bright field images; *left panel*, fluorescence images. **b** Immunofluorescence using anti-acetylated histone H4K5ac antibodies in isolated mouse round and elongated spermatids. In negative controls, the primary antibodies were replaced by rabbit IgG. *PS/RS* pachytene spermatocyte/round spermatids, *ES* elongated spermatid

Next, we analyzed the presence of histone methylation in mature human sperm (Fig.1a). Histone methylation is a well-characterized modification that has been associated with both transcriptionally permissive and restrictive chromatin configurations. Surprisingly, we observed that 12 to 30 % of the nuclei in normal sperm contain a heterogeneous distribution

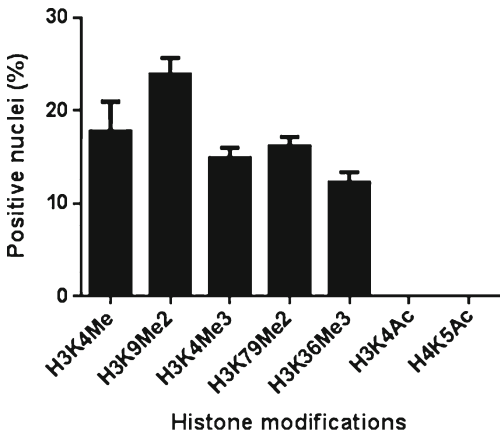

Fig. 2 Quantification of the percentage of human sperm nuclei with positive staining for the different histone marks. Results are expressed as mean ± S.E.M

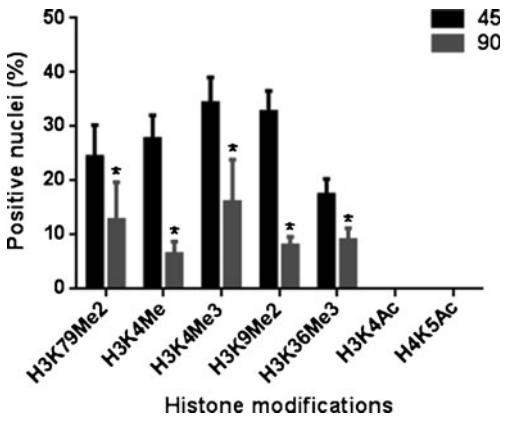

Fig. 3 Quantification of the percentage of human sperm nuclei with positive staining for the different histone marks in the different subpopulations of isolated sperm after discontinuous gradient centrifugation (L45 and L90). Results are expressed as mean ± S.E.M. *Asterisks* denotes statistical significance compared with L45 ($P<0.05$). ($n=6$)

of the marks H3K4Me1, H3K9Me2, H3K4Me3, H3K79Me2, and H3K36Me3 (Fig. 2).

In order to evaluate whether the heterogeneous distribution of these histone marks in mature human sperm is associated with the functional and morphological quality of sperm, we next performed a discontinuous gradient separation of spermatozoa with high and low motility [7] and proceeded to perform immmunostaining for all the histone marks previously mentioned. As shown in Fig. 3, the percentage of sperm with histone marks increased significantly ($P<0.05$) in the population with low motility (L45) compared to the high motility population (L90). As indicated above, we did not observe histone acetylation in either sperm subset.

Discussion

Histone modifications are one of the most thoroughly studied areas in epigenetics due to their importance in transcriptional regulation of gene expression. Normally, around 10–15 % of the nuclear proteins in mature human sperm are histones. Because certain subfertile or infertile individuals displayed abnormal levels of histones due to an abnormal protamine to histone exchange, we decided to include only samples that displayed normal levels of protamines and histones in their nuclei as evaluated by aniline blue staining [8]. The composition of the histone post-translational modifications that persist in the nucleosomes of mature human sperm is largely unknown. In this study we assessed the levels of several histone methylations (H3K4Me, H3K4Me3, H3K9Me2, H3K79Me2, H3K36Me3) and acetylations (H3K4Ac and H4K5Ac) in normal and abnormal human sperm. In this regard, we evaluate in a group of normal individuals (according to the WHO criteria) the presence of these histone marks in isolated sperm nuclei.

Histone acetylation occurs during spermiogenesis prior to the exchange of histone by protamines. The massive acetylation creates a less stable chromatin that facilitates the accessibility of proteins that participate in the exchange [12]. This transient acetylation is lost after completion of the exchange. Thus, we hypothesize that this massive histone acetylation is not present in mature sperm but may remain present in those situations where the normal spermiogenesis process is perturbed resulting in the presence of abnormal cells in the ejaculate. In this regard, it has been shown that in the human ejaculate coexist different sperm subsets that vary in terms of sperm function (i.e., in their capacity to undergo protein tyrosine phosphorylation during capacitation), maturational stage and morphology [12]. These sperm subsets can be separated and isolated by discontinuous gradient centrifugation, where the highest quality sperm are recovered from the pellet of the gradient (L90), while the lowest quality sperm with poor motility and morphology remain in the upper layers of the gradient (L45). However, we did not observe histone acetylation (H3K4Ac and H4K5Ac) in normal or abnormal mature human sperm. This result indicates that histone acetylation does not persist in human sperm after ejaculation.

Histone methylation is one of the most studied epigenetic modification. Unexpectedly, we observed an heterogeneous distribution of the modifications H3K4Me1, H3K9Me2, H3K4Me3, H3K79Me2, and H3K36Me3. In this case, when we compared the best quality and the worst quality spermatozoa (represented by the high and low motility fraction of the discontinuous density gradient respectively), we observed remarkable differences between both fractions. This difference may be related to abnormal levels of histone in the poor motility fractions due to defective exchange by protamines during spermiogenesis, as observed in certain sperm pathological conditions.

Methylation of histones in lysines 3 or 4 have been associated with either activation or repression of transcription, respectively [13]. Some of these histone marks have been shown to participate in several processes in the male reproductive tract, such as gene expression during spermatogenesis, differentiation of spermatogonia into spermatocytes [14], and X-chromosome inactivation [15] among others. A question that remains to be answered is whether the histone methylation present in mature sperm remains associated with the paternal genome after the protamine-histone exchange that takes place immediately after fertilization and if these have a function during early premiplantation embryo development. After fertilization all the protamines and almost all the paternal histones are degraded and replaced by oocyte histones. However, based on ethical grounds, the fate of these marks in human preimplantation embryos cannot be assessed unless these embryos are discarded because of multinucleation, fragmentation or developmental arrest. Nevertheless, these are pathological conditions where these events may be altered.

In summary, we report the heterogeneous presence of histone modifications in human sperm. The presence of these marks is higher in poor functional quality sperm, suggesting that defects in the process of spermatogenesis may alter the correct epigenetic programing in mature sperm. Further studies are required to evaluate the impact of these observations in infertile individuals.

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